Slov Vet Res
DOI 10.26873/SVR-2053-2024
UDC 001.891:577.171.6:578:615.2:616.523:636.4
Pages: 81–4

Human Herpesvirus Epstein-Barr (EBV) and Its Porcine Homologs Unveil the Conserved Mechanism of Receptor Endocytosis: New Insights Into Viral Immune Evasion and Antiviral Therapy Potential?

Humani herpesvirus Epstein-Barr (EBV) in njegovi prašičji homologi razkrivajo ohranjeni mehanizem receptorske endocitoze: nov vpogled v virusno izmikanje imunskemu sistemu in potencialne protivirusne terapije?

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Accepted: 19 May 2024

Over the past two years, the University of Ljubljana and the Republic of Slovenia's public agency, the Slovenian Research and Innovation Service (ARIS), have acknowledged and celebrated several exceptional accomplishments in viral receptor research. These achievements are considered among the finest by both the University of Ljubljana and ARIS. The significance of these achievements lies in the research findings, which indicate that the EBV-BILF1 receptor encoded by the Epstein-Barr virus (EBV) could become a promising new drug target for EBV. Additionally, the research suggests pigs represent a great model for further investigations (1—4).

Epstein-Barr virus (EBV), also known as human herpesvirus 4 (HHV4), is found in about 95% of the world's adult population and causes infectious mononucleosis (5). EBV is also an oncovirus and is associated with various types of lymphoma and other cancers. In patients with compromised immune system following solid organ transplants (SOT) and hematopoietic stem cell transplants (HSCT) EBV infection is considered as a driving factor for the development of post-transplant lymphoproliferative disorder (PTLD), which leads to various types of tumours with a high risk of fatal outcome (6,7).

Univerza v Ljubljani in Javna agencija za znanstvenoraziskovalno in inovacijsko dejavnost Republike Slovenije (ARIS) sta v preteklih dveh letih prepoznali in počastili več izjemnih dosežkov na področju raziskovanja virusnih receptorjev. Ti dosežki – tako na Univerzi v Ljubljani kot na ARIS-u – sodijo med najboljše. Njihov pomen izhaja iz raziskav, ki kažejo na to, da bi se lahko receptor EBV-BILF1, kodiran na virusu Epstein-Barr (EBV), uporabljal kot obetavna nova tarča za zdravljenje EBV. Poleg tega raziskava predlaga uporabo prašičev kot modela za nadaljnje preiskave (1—4).

Virus Epstein-Barr (EBV), poznan tudi kot humani herpesvirus 4 (HHV4), je prisoten pri približno 95 odstotkih odraslega svetovnega prebivalstva in povzroča infekcijsko mononukleozo (5). EBV je tudi onkovirus in je povezan z različnimi vrstami limfoma in drugimi vrstami raka. Pri bolnikih z ogroženim imunskim sistemom po presaditvi trdnih organov (SOT) in presaditvi hematopoetskih matičnih celic (HSCT) okužba z EBV šteje za vodilni dejavnik za razvoj posttransplantacijske limfoproliferativne motnje (PTLD), ki vodi do različnih vrst tumorjev z visokim tveganjem za usoden izid (6, 7).

To achieve such severe complications many herpesviruses, including EBV, human cytomegalovirus (HCMV) and Kaposi's sarcoma-associated virus (KSHV), have developed various strategies to infect and persist in the host for a lifetime (8,9). One of these strategies is the expression of viral G protein-coupled receptors (vGPCRs), which are structurally and functionally similar to host GPCRs. Viral GPCRs play a crucial role in immunological processes within the cell. They were probably acquired from the host by molecular piracy and are now exploited by the virus to manipulate and evade the host's immune response. Thus, viral GPCRs are considered important drug targets for various diseases, therefore research of the pharmacological properties of these receptors and their mechanisms of endocytosis is a crucial step in their identifications as drug targets (10).

EBV encodes a viral G-protein-coupled receptor (vGPCR) called EBV-BILF1, which plays a crucial role in oncogenesis and immune evasion. EBV-BILF1 orthologs in pigs are encoded by three porcine lymphotropic herpesviruses (PLHV1—3) (11). The recent unveiling of the EBV-BILF1 structure by an international team, including Slovenian scientists, who described the specific features and highly conserved constitutive activity for Gαi coupling in a ligand-independent fashion, promises a significant advance in the formulation of strategies targeting the BILF1 receptors (3).

Endocytosis plays a crucial role in cellular transport of GPCRs. Several herpesviruses, including EBV, encode vGPCRs that help to evade the immune system and virus to spread. One of the published articles focused on the endocytosis of vGPCRs and their importance, highlighting the constitutive internalization of BILF1 from human and porcine γ-1 herpesviruses. New methods, such as real-time fluorescence assays, have played an important role in the study of these processes (1,2,4).

Furthermore, mechanisms of the internalization of the EBV-BILF1 receptor and receptor orthologs from porcine lymphotropic herpesviruses (PLHVs) were studied in detail in comparison to EBV-BILF1. A real-time fluorescence resonance energy transfer (FRET) assay together with the expression of dominant-negative variants of specific endocytic proteins, including dynamin-1 and the clathrin inhibitor Pitstop2 was used to investigate the mechanism of EBV-BILF1 internalization. Bioluminescence resonance energy transfer (BRET) saturation analysis was used to determine the interactions of the BILF1 receptors with β-arrestin2 and Rab7. Finally, the informational spectrum method (ISM) was used for bioinformatics analysis to investigate the interaction affinity of BILF1 receptors with various cellular components. Overall, this work showed that all BILF1 receptors undergo dynamin-dependent, clathrin-mediated constitutive endocytosis, that also dependent on caveolin-1 which is critical for proper BILF1 receptor trafficking. After receptor internalization recycling and degradation pathways have been proposed for BILF1 receptors. The study provides new insights into receptor transport and the similarities between the internalization mechanisms

Da bi dosegli tako resne komplikacije, so številni herpesvirusi, vključno z EBV, humanim citomegalovirusom (HCMV) in virusom, povezanim s Kaposijevim sarkomom (KSHV), razvili različne strategije za okužbo in vztrajanje v gostitelju vse življenje (8, 9). Ena od strategij vključuje izražanje virusnih s proteini G sklopljenih receptorjev (vGPCR-jev), ki so strukturno in funkcionalno podobni GPCR-jem gostitelja. vGPCR-ji igrajo ključno vlogo predvsem pri imunskih procesih v celici. Verjetno so jih od gostitelja pridobili s pomočjo molekularnega piratstva, sedaj pa jih virus izkorišča za manipulacijo in izogibanje imunskemu odzivu gostitelja. Tako vGPCR-ji veljajo za pomembne tarče zdravil za različne bolezni, zato je raziskava farmakoloških lastnosti teh receptorjev in njihovih mehanizmov endocitoze pomemben korak pri njihovi identifikaciji kot tarč zdravil (10).

EBV kodira z G-proteini sklopljen receptor (vGPCR), imenovan EBV-BILF1, ki je ključen pri onkogenezi in izmikanju imunskemu sistemu človeka. Ortologe receptorja EBV-BILF1 pri prašičih kodirajo trije gamaherpesni prašičji virusi (PLHV1—3) (11). Nedavno odkritje strukture receptorja EBV-BILF1, pri katerem so bili vključeni tudi slovenski raziskovalci, ki opisuje njegove strukturne značilnosti in visoko ohranjeno konstitutivno aktivnost za vezavo z Gαi podenoto proteina G v odsotnosti liganda, bo znatno pripomoglo k napredku pri raziskovanju receptorjev BILF1 kot obetajočih tarč za nova zdravila (3).

Endocitoza je pomembno vpletena v znotrajcelični transport, tudi GPCR-jev. Različni herpesvirusi, vključno z EBV, ki kodirajo vGPCR-je, pomagajo pri izogibanju imunskemu sistemu in širjenju virusa. Objavljeni pregledni članek obravnava endocitozo vGPCR-jev in njen pomen, s poudarkom na konstitutivni internalizaciji človeških in prašičjih γ-1 herpesvirusnih receptorjev BILF1. Nove metodologije, kot so metoda internalizacije v realnem času, ki temelji na metodi FRET, so bile ključne pri proučevanju teh procesov (1, 2, 4).

V enem izmed člankov, ki so jih objavili slovenski raziskovalci v sodelovanju s partnerji v tujini, so bili podrobno proučevani mehanizmi za internalizacijo receptorja EBV-BILF1 in translacijski potencial ortologov receptorja EBV-BILF1, ki jih kodirajo prašičji limfotropni herpesvirusi (PLHV) v primerjavi z EBV-BILF1. Za raziskovanje mehanizma internalizacije receptorjev BILF1 je bila uporabljena nova metoda, temelječa na fluorescenčnem prenosu resonančne energije v realnem času (FRET) skupaj z izražanjem dominantno negativnih mutant specifičnih proteinov, vključenih v proces endocitoze, vključno z dinaminom-1 in zaviralcem klatrina Pitstop2. Saturacijska analiza bioluminiscenčnega prenosa resonančne energije (BRET) je bila uporabljena za preučevanje interakcij receptorjev BILF1 z β-arestinom2 in Rab7. Poleg tega je bila za bioinformatično analizo uporabljena metoda informacijskega spektra (ISM) z namenom raziskovanja interakcijske afinitete receptorjev BILF1 z različnimi celičnimi proteini ali pododdelki. Študija je pokazala, da je endocitoza vseh receptorjev BILF1 konstitutivna in odvisna od dinamina

of EBV-BILF1 and PLHV1—2 BILF1 suggest potential translational applications for PLHVs (1).

Another study focused on the constitutive activity of EBV-BILF1 and its role in EBV-mediated immunosuppression and oncogenesis. The cryo-electron microscopy structure, resolved at 3.2 Å, showed that an extracellular loop within EBV-BILF1 obstructed the usual chemokine binding site, indicating EBV-BILF1 receptor activation without ligand, suggesting that the intrinsic activity of EBV-BILF1 underlies immunosuppression and virulence without being dependent on the presence of a ligand. This finding has implications in discovering novel functions of GPCRs encoded by similar viruses and for the development of antiviral therapies (3).

EBV-BILF1 has also been shown to play a critical role in oncogenesis and immune evasion by downregulating major histocompatibility complex (MHC-I) molecules in infected cells. This downregulation is thought to occur through the internalization of EBV-BILF1 together with MHC-I. In immunosuppressed transplant patients, EBV infection can lead to PTLD. Miniature pigs infected with PLHV1—3 develop a similar disease, which makes them potential preclinical model for PTLD. BILF1 orthologs encoded by PLHVs have similar characteristics to EBV-BILF1, including cell surface localization, internalization, MHC-I downregulation, and Gαi signaling patterns. PLHV1 was observed in the lymphoid tissues of pigs suffering from PTLD, indicating its involvement in PTLD infection. The lack of preclinical models to validate BILF1 receptors and study EBV-related diseases is currently a challenge. Nevertheless, the results of these recently published articles suggest that PLHV1-infected pigs may represent a viable model for studying the potential role of BILF1 as a key driver and therapeutic target in EBV-associated proliferative diseases (2).

As different efforts are made to find a new therapeutic possibility against EBV-related diseases recent paper in Science (12) pronounced the importance of finding new therapeutic possibilities against EBV-related diseases. In this paper latent gene EBNA-2, which initiates the transcription of viral and cellular genes and induces B-cell transformation and was exploited many years in connection with vGPCRs (13) was being shown to be involved in expression of metabolic enzyme IDO1 in infected cells. IDO1 controls immune responses and controls *de novo* synthesis of NAD+. Blocking IDO1 could therefore for be used as the first precision medicine cellular-metabolic intervention affecting viral infection *in vivo*.

This research is not only pushing the boundaries of what is known about EBV and developing this vital research area, but it is also attracting international acclaim. This work is attracting prestigious and ongoing collaborators from Denmark, Germany, Serbia, the United Kingdom and the United States, such as Stanford and Colorado Universities. The featured studies provided a comprehensive understanding of the role of EBV-BILF1 in viral pathogenesis and immune evasion as

ter poteka preko klatrinsko odvisne poti. Afiniteta interakcije med receptorji BILF1 in kaveolinom-1, skupaj z zmanjšano internalizacijo ob prisotnosti dominantno negativne variante kaveolina-1, je pokazala vpletenost kaveolina-1 v prerazporejanje receptorjev BILF1. Po internalizaciji so bile pri receptorjih BILF1 proučevane poti njihovega recikliranja in razgradnje. Študija odstira nov vpogled v znotrajcelično prerazporejanje receptorjev, podobnosti mehanizmov internalizacije med EBV-BILF1 in PLHV1—2 BILF1 pa kažejo na morebitne translacijske aplikacije za PLHV (1).

Naslednja študija se je osredotočala na konstitutivno aktivnost receptorja EBV-BILF1 in njegovo vlogo pri zaviranju delovanja imunskega sistema in pri virusni onkogenezi EBV. Struktura, pridobljena z uporabo krioelektronske mikroskopije pri ločljivosti 3,2 Å, je pokazala, da zunajcelična zanka receptorja EBV-BILF1 ovira običajno vezno mesto za kemokine, kar kaže na aktivacijo receptorja v odsotnosti liganda in pomeni, da aktivnost receptorja EBV-BILF1 vpliva na zaviranje imunskega sistema gostitelja in virulenco virusa, brez vezave liganda na receptor. Ta ugotovitev ima pomembne posledice za delovanje GPCR-jev, kodiranih s podobnimi virusi, in za razvoj terapij proti EBV (3).

Kot je bilo omenjeno, je receptor EBV-BILF1 ključen pri onkogenezi in izmikanju imunskemu sistemu, kar doseže z znižanjem površinske izraženosti glavnih molekul histokompatibilnega kompleksa (MHC-I) na okuženih celicah. Do znižane površinske izraženosti naj bi prišlo zaradi internalizacije receptorja EBV-BILF1 skupaj z molekulami MHC-I. Pri bolnikih z zavrtim imunskim sistemom po presaditvi tkiv lahko okužba z EBV povzroči posttransplantacijsko limfoproliferativno bolezen (PTLD). Miniaturni prašiči, okuženi s prašičjim limfotropnim herpesvirusom (PLHV1—3), razvijejo podobno bolezen, zaradi česar so potencialno zanimivi kot predklinični modeli za PTLD. Ortologi BILF1, kodirani na virusih PLHV, kažejo podobne lastnosti kot EBV-BILF1, vključno z lokalizacijo na celični površini, internalizacijo, znižano regulacijo molekul MHC-I in znotrajceličnim prenosom preko Gαi. PLHV1 so izsledili v limfatičnem tkivu prašičev, obolelih s PTLD, kar kaže na njegovo vpletenost v okužbo s to boleznijo. Kljub temu pomanjkanje predkliničnih modelov za validacijo receptorjev BILF1 in preučevanje bolezni, povezanih z EBV, predstavlja trenutni izziv. Vendar pa ugotovitve v nedavno objavljenih člankih razkrivajo, da bi lahko bili prašiči, okuženi s PLHV1, obetajoč model za raziskovanje potencialne vloge EBV-BILF1 kot ključnega igralca in terapevtske tarče pri proliferativnih motnjah, povezanih z EBV (2).

Veliko raziskovalnih skupin si prizadeva poiskati nove terapevtske možnosti proti boleznim, povezanim z EBV. Eden izmed prebojnih je nedavni članek, objavljen v Science (12), ki je poudaril pomen iskanja novih terapevtskih možnosti proti boleznim, ki so povezane z EBV. V tem prispevku je bilo dokazano, da je latentni gen EBNA-2, ki sproži transkripcijo virusnih in celičnih genov ter transformacijo B-celic in so ga mnogo let proučevali v povezavi z vGPCR (13), vključen v

well as novel insights into potential therapeutic strategies targeting this receptor.

Funding: We gratefully acknowledge financial support from the Slovenian Research Agency (grant number P4-0053) and a PhD grant awarded to MM. Furthermore, we acknowledge the funding received from the Slovenian-Serbian bilateral project (BI-RS/20-21-045) and the Ministry of Science, Technological Development, and Innovation of the Republic of Serbia (grant number 451-03-47/2023-01/200017).

Acknowledgments: Prof. Dr. Dr. Catrin S. Rutland is deeply thanked for her assistance with language editing.

izražanje presnovnega encima IDO1 pri okuženih celicah. IDO1 nadzoruje imunske odzive in *de novo* sintezo NAD+. Blokiranje IDO1 bi se torej lahko uporabilo kot prva usmerjena medicinska celično-presnovna intervencija, ki bi vplivala na virusno okužbo *in vivo*.

Opisane raziskave premikajo meje znanega o EBV in razvijajo to ključno raziskovalno področje, hkrati pa so tudi mednarodno odzivne in omogočajo sodelovanje s prestižnimi sodelavci iz Danske, Nemčije, Srbije, Združenega kraljestva in Združenih držav, kot sta Univerza Stanford in Univerza v Koloradu. Nominirane in nagrajene študije omogočajo celovitejše razumevanje vloge receptorja EBV-BILF1 pri virusni patogenezi in izogibanju imunskemu sistemu ter vpogled v možne terapevtske strategije, ki ciljajo ta receptor.

Financiranje: Zahvaljujemo se Javni agenciji za znanstvenoraziskovalno in inovacijsko dejavnost Republike Slovenije (ARIS) za financiranje programa P4-0053 in mlade raziskovalke MM ter za sredstva iz slovensko-srbskega bilateralnega projekta (BI-RS/20-21-045) in z Ministrstva za znanost, tehnološki razvoj in inovacije Republike Srbije (št. projekta 451-03-47/ 2023-01/200017).

Zahvala: Zahvaljujemo se dr. Andreji Jezernik za pregled slovenskega besedila.

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